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Award Number: DAMD17-98-1-8514

TITLE: Oral Contraceptives Use by Young Women Reduces Peak Bone Mass

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REPORT DATE: December 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2002	Final (1 Sep 98 - 30 Nov 02)

4. TITLE AND SUBTITLE
Oral Contraceptives Use by Young Women Reduces Peak Bone Mass

5. FUNDING NUMBERS
DAMD17-98-1-8514

6. AUTHOR(S) :
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The goal of these studies was to determine the role that hypoandrogenemia plays in the effects of oral contraceptives (OC) on bone metabolism and peak bone mass (PBM) in young female rats. Adolescent/young adult Sprague-Dawley rats were treated with 1) placebo, 2) OC, 3) OC supplemented with an androgen (methyltestosterone), or 4) an anti-androgen (bicalutamide) to determine the potential role that suppression of androgens plays on bone metabolism, bone architecture, and the attainment of PBM. Our specific aims were to determine:

1. If oral contraceptive steroid (OC) treatment leads to decreased peak bone mass in young intact female rats. *Findings: OC use decreased the peak bone mass of young intact female rats.*
2. If the addition of a non-aromatizable androgenic steroid to OCs prevents the detrimental effects of OC use on peak bone mass. *Findings: The non-aromatizable androgenic steroid did not prevent the adverse effects of OCs to the growing skeleton of young rats at the dose used.*
3. If anti-androgen treatment mimics the effect of OC use on peak bone mass. *Findings: The anti-androgen used did not mimic the adverse effect of OCs on the growing skeleton of young rats.*

14. SUBJECT TERMS	15. NUMBER OF PAGES
peak bone mass, oral contraceptives, osteoporosis, androgens, estrogen	29
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Oral contraceptive (OC) treatment of young adult female non-human primates has been shown to cause reductions in bone mass gain compared to a control group of animals (Register et al., 1997). The mechanism(s) underlying this effect are not known, although one of the well known side effects of OC is hypoandrogenemia. The central hypothesis underlying the proposed study was that oral contraceptive (OC) treatment of adolescent and young adult females causes an abnormal depression of circulating androgens which results in a depression of bone gain during this critical period. The end result may be a reduction in peak bone mass and an increased risk of stress fractures and osteoporosis in long term OC users. Similar results might be observed by suppression of androgen activity in intact animals in the absence of OC therapy. Conversely, supplementation of OC-treated females with an androgen might result in restoration of normal bone mineral accretion. The overall goal of the proposed study was to determine the role of hypoandrogenemia in the effects of OC on bone metabolism and on peak bone mass in young female rats. A secondary goal was to also explore alternative mechanisms which might be responsible for this phenomenon. For these studies, we used Sprague-Dawley rats, a well-characterized animal model of ovarian hormone effects on bone metabolism. These animals were examined while in the adolescent and young adult age range. We treated intact animals with 1) Placebo, 2) OC, 3) OC supplemented with an androgen (methyltestosterone), or 4) Anti-androgen therapy (bicalutamide) to determine the potential role that suppression of androgens plays on bone metabolism, bone architecture, and the attainment of PBM.

BODY

Personnel

- Dr. Manuel Jayo, Senior Pathologist with Pathology Associates International and adjunct Associate Professor of Pathology at Wake Forest University School of Medicine.
- Dr. Erni Sulistiawati, an Indonesian D.V.M. and a Ph.D. candidate enrolled at the Institut Pertanian Bogor (IPB). October 1, 1998 - Aug 30, 1990. Dr. Sulistiawati's mentor in Indonesia was Dr. Dondin Sajuthi.
- Dr. Uriel Blas-Machado, post-doctoral fellow. September 1, 1998 to June 11, 1999. Dr. Blas-Machado's salary was supported by a Training Grant from the NCRR, NIH.
- Ashley Albers, summer student enrolled in Salem College (Winston-Salem, NC). June-July 2002.
- Technical assistance was provided by Mrs. Pam Louderback, Mr. Sam E. Rankin, and Mr. Gerald Perry.

Experimental Procedures and Outcomes

- Seven rats (10% of total approved by the institutional Animal Care and Use Committee [ACUC]) were used to conduct the pilot project (TABLE 1). This project allowed us to test the palatability and the feasibility of procedures (sedation, bleeding, densitometry, etc) to be conducted in the live animals.

Table 1. PILOT PROJECT

Exp Time	*Week*	*Age (days)*	*Comment*	*Date*
-1	1	63		26-Oct-98
Start diet	0	70	DXA 1	02-Nov-98
1	3	77		09-Nov-98
2	4	84	DXA 2	16-Nov-98

Based on our previous work with non-human primates (Register et al., 1997), the food consumption and body weight gains during the pilot project, no additional palatability issues were considered and the go ahead for the proposed experiment was given.

- As part of the annual review, on October 19, 1998, the ACUC Protocol A97-147 was approved for extension until October 20, 1999.

- Semipurified food (with hormones) was prepared (Table 2) and keep frozen until ready to use. Once open, it was kept refrigerated.

Table 2. Semi-purified diet, designed to contain no isoflavones. Each 100 g of semi-purified high-fat diet contained the following products.

Food	***(g)***
Casein, USP	10.5
Lactalbumin	10.0
Dextrin	30.6
Sucrose	28.0
Alphacel	10.0
Lard	5.20
Safflower Oil (linoleic)	1.00
Choline Bitartrate	0.20
Vitamin Mixture, AIN-76A	1.00
Mineral Mix, AIN-76	3.50

- In contrast to our pilot information from rats and our previous monkey data, we found the rats were not eating as expected (Table 3). After review, feed was to be produced every other week to maintain palatability. The differences in total consumption were dramatic, on average 3 to 4 g of food per day were not consumed by the OC and OC+MT groups (Table 3).

Table 3. Average (AVE, g) feed consumption per day during the experiment

Group	**AVE ± SD**
Control	20.02 ± 1.94
OC	16.79 ± 4.80
OC+MT	17.09 ± 5.16
Cas	19.35 ± 2.09

- The average feed consumption varied with the contraceptive schedule (3 days on and 1 day off, to mimic a woman's pill cycle) as shown in Table 4:

Table 4. Average (g) feed consumption per day-cycle

Group	Day 1	Day 2	Day 3	Day 4 (*NO STEROIDS*)
Control	19.71	20.18	19.79	20.42
OC	12.49	15.03	15.39	24.29
OC+MT	11.96	15.41	16.46	24.53
Cas	18.91	19.47	18.99	20.03

- Two fluoroscein bone labels were ordered and given (demeclocycline and calcein) prior to necropsy.

- Necropsies and collection of tissues were carried out in March 1999. Type and number of tissues per animal collected processed, sectioned, stained (H&E), and histologically evaluated included: ovaries (2), uterus and horns (2), vagina, cervix and urinary bladder (2), liver lobes (3), spleen and kidneys (3), adrenal glands (2), thyroids, thymus, and pancreas (3), heart (2), lungs (2), brain (2), mammary gland (2), pituitary (1), left femur (1) and L2 vertebra (1).

- At necropsy, the right tibia and L3 vertebrae were collected, the soft tissue cleaned, and the bones placed in dark-brown stained 30 ml glass bottles containing 70% alcohol (ETOH). The right tibia's tuberosity was shaved with a sharp scalpel blade for proper fixation and the dorsal arches of the lumbar L3 vertebra removed.

- Bones were packaged and sent to Pathology Associates International (PAI) in Frederick, MD for plastic bone histologic processing. *Bone Histomorphometry:* PAI processed, embedded in methyl methacrylate (MMA), and sectioned at 5-10 μm, and mounted unstained or stained sections of bone with modified tetrachrome with Von Kossa method. *Standard histomorphometry*: The abbreviations used were based on the ASBMR standard nomenclature (1). Structural and dynamic parameters were measured.

- Soft and hard tissues were fixed, processed, embedded, section and stained for evaluations by Drs. Jayo and Blas-Machado.

***Ex vivo* primal and distal pQCT scanning of tibia**

Methods After necropsy, the right tibia was kept frozen at −20°C until scanned using peripheral quantitative computed tomography (pQCT). The Norland Stratec XCT960 pQCT Bone Densitometer (Ft. Atkinson, WI) was used for pQCT measurements. Although methodology differed slightly from other reports, precision was similar to that previously reported (Gasser 1995, Sato 1997). A voxel size of 0.148 mm and a threshold for cortical bone of 500 was selected throughout the scans (Contour Mode 1, Peel mode 2, Cortical mode 4). Scans were taken at the proximal (metaphyseal and cancellous rich) and distal (primarily cortical) portions of the tibia. Based on previous reports and histological evaluations, pQCT scans were taken for proximal tibia at a constant 5 mm distance from the knee joint. Distal tibia evaluations were taken at a constant 1 mm proximal to the fibulo-tibial junction. For both sites, measurements included Cancellous Bone Mineral

Content (Cn.BMC, in mg/mm [trab_cnt]), Cancellous Bone Mineral Density (Cn.BMD, in mg/mL [trab_dn]), Cancellous Bone Area, (Cn.B.Ar, in mm^2, [trab_a]), Cortical Bone Mineral Content (Ct. BMC, in mg/mm, [crt_cnt]), Cortical Bone Mineral Density (Ct.BMD, in mg/mL, [crt_den]), Cortical Bone Area, (Ct.B.Ar, in mm^2, [crt_a]), Cortical Thickness (Ct.Th., mm, [crt_thk]), Periosteal perimeter (Ps.Pm, mm, [peri_c]), Endosteal Perimeter (Ec.Pm, mm, [endo_c]), Polar Moment of Inertia (P.M.I., mm^4, [ip_cm_w]), and Moment of Resistance or the (P.M.R., mm^3, [rp_cm_w]).

Statistics All QCT raw data is expressed as mean ± SEM (Table 5). All statistical analyses were conducted using version 7.0 BMDP Statistical Software (Los Angeles, CA). Data was subjected to one-way analysis of variance (ANOVA) and post hoc pairwise comparisons utilizing Tukey's test. The letter symbol in all tables and graphs indicate the level of significance compared to Control animals ($^a p<0.05$; $^b p<0.01$).

Table 5. pQCT measurements taken from the right proximal tibia of young female rats at a constant 5 mm distal site from the joint space.

Parameter	*Control*	*OC*	*OC+MT*	*Casodex*	*p-value*
N	14	14	14	12	x
Cn.BMC	1.10 ± 0.15	1.46 ± 0.08	**1.61 ± 0.06^b**	1.11 ± 0.14	0.0023
Cn.BMD	308 ± 9.04	**270 ± 9.77^a**	**254 ± 10.8^b**	305 ± 6.40	0.0002
Cn.B.Ar	3.72 ± 0.55	5.50 ± 0.39	6.46 ± 0.32	3.68 ± 0.48	0.0000
Ct. BMC	9.65 ± 0.25	**7.74 ± 0.19^b**	**7.56 ± 0.23^b**	9.34 ± 0.23	0.0000
Ct.BMD	922 ± 13.31	920 ± 9.56	917 ± 10.67	909 ± 19.30	NS
Ct.B.Ar	10.5 ± 0.33	**8.42 ± 0.24^b**	**8.24 ± 0.20^b**	10.3 ± 0.39	0.0000
Ct.Th	0.78 ± 0.03	**0.65 ± 0.01^b**	**0.63 ± 0.02^b**	0.77 ± 0.02	0.0000
Ps.Pm	15.9 ± 0.22	**14.9 ± 0.22^b**	**15.0 ± 0.16^a**	15.9 ± 0.26	0.0012
Ec.Pm	11.0 ± 0.26	10.8 ± 0.20	11.1 ± 0.17	11.0 ± 0.24	NS
P.M.I.	36.7 ± 1.29	**27.6 ± 1.26^b**	**27.3 ± 1.05^b**	35.8 ± 1.31	0.0000
P.M.R	11.3 ± 0.34	**8.90 ± 0.36^b**	**8.78 ± 0.33^b**	11.1 ± 0.41	0.0000

Results The QCT-derived parameters measured at the distal tibia (primarily cortical bone) were not significantly different among groups (data not shown). However, significant differences between Control vs OC or OC+MT treated animals were detected at the proximal tibia in both cortical and cancellous parameters (Table 1). None of the measurements were significantly different between Control and Casodex groups. *

Conclusions OC use in growing rats, at a dose which corresponds to a 25% lower dose than that recommended for contraception in women, caused bone deficits at the proximal tibia compared to Control animals. This bone deficit was not prevented by OC supplemented with the androgen methyltestosterone. Surprisingly, and in contrast to previous reports (Lea et al., 1996), the nonsteroidal anti-androgen bicalutamide (Casodex) ingestion in growing rats did not cause significant bone changes compared to Control rats. Dose and route of administration were different in the two studies, as Lea et al. (1996) administered Casodex SQ daily ti rats at 20 mg/kg/day for 21 days (420 mg total) while in the present study Casodex was given orally at a dose corresponding to a human dose of 50 mg/day (0.89 mg/100 g of BW in the rat) for 105 days.

Table 6. **Histomorphometric measurements for the distal femur metaphysis. Note: The total of distal femur metaphysis (bone + marrow) was identical for all groups (3.73 mm^2).**

Parameter	*Group*	*Mean*	*SEM*	*P-value*
BV	OC	0.94	0.11	0.000
	Control	1.22	0.11	
	OC+MT	0.70	0.07	
	Casodex	1.30	0.13	
BS	OC	24.63	1.75	0.001
	Control	28.65	1.13	
	OC+MT	21.20	1.27	
	Casodex	30.34	2.23	
BV/TV	OC	25.24	2.84	0.000
	Control	32.68	2.96	
	OC+MT	18.70	1.77	
	Casodex	34.83	3.37	
Tb.Th.	OC	57.25	3.25	0.005
	Control	65.07	3.89	
	OC+MT	50.44	2.24	
	Casodex	65.05	3.26	
Tb.N.	OC	4.20	0.30	0.001
	Control	4.89	0.19	
	OC+MT	3.62	0.22	
	Casodex	5.18	0.38	
Tb.Sp.	OC	204.75	29.72	0.059
	Control	143.93	12.51	
	OC+MT	240.16	20.37	
	Casodex	158.23	42.43	

Soft tissue histologic evaluations

Ovaries- Ovaries were evaluated by counting the number of primary, growing, and antral follicles. Corpora lutea (CL) were counted and classified into atretic CL, hemorrhagic CL, and mature CL.

Table 7.

Primary (ANOVA p=0.260)

	OC	Control	OC+MT	Casodex
N	13	14	14	12
Mean	31.615	26.000	35.000	21.833
STD	17.868	19.896	20.840	10.338
SEM	4.956	5.317	5.570	2.984
Min	82.000	69.000	74.000	35.000
Max	11.000	4.000	5.000	6.000

Growing (ANOVA p=0.312)

	OC	Control	OC+MT	Casodex
N	13	14	14	12
Mean	7.000	4.786	6.214	4.417
STD	3.851	3.043	3.641	3.397
SEM	1.068	0.813	0.973	0.981
Min	14.000	9.000	15.000	11.000
Max	3.000	0.000	2.000	0.000

Antral (ANOVA p=0.448)

	OC	Control	OC+MT	Casodex
N	13	14	14	12
Mean	12.077	13.143	9.357	11.750
STD	5.283	7.833	5.733	5.895
SEM	1.465	2.094	1.532	1.702
Min	22.000	26.000	19.000	24.000
Max	5.000	4.000	0.000	1.000

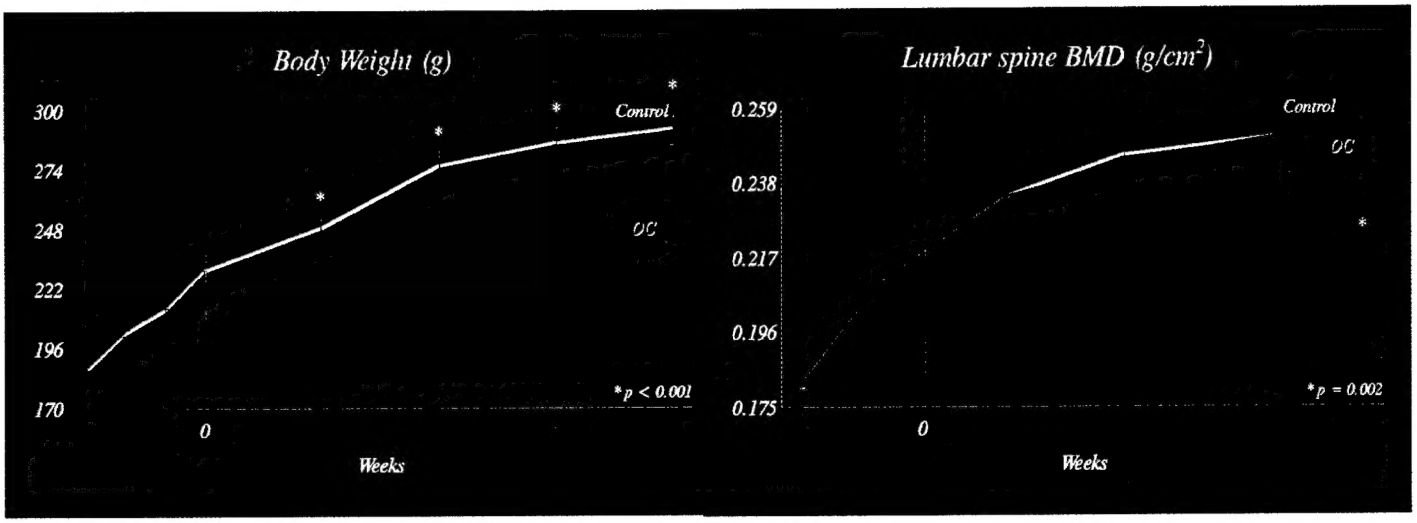
Atretic (ANOVA p=0.823)

	OC	Control	OC+MT	Casodex
N	13	14	14	12
Mean	12.077	13.143	9.357	11.750
STD	5.283	7.833	5.733	5.895
SEM	1.465	2.094	1.532	1.702
Min	22.000	26.000	19.000	24.000
Max	5.000	4.000	0.000	1.000

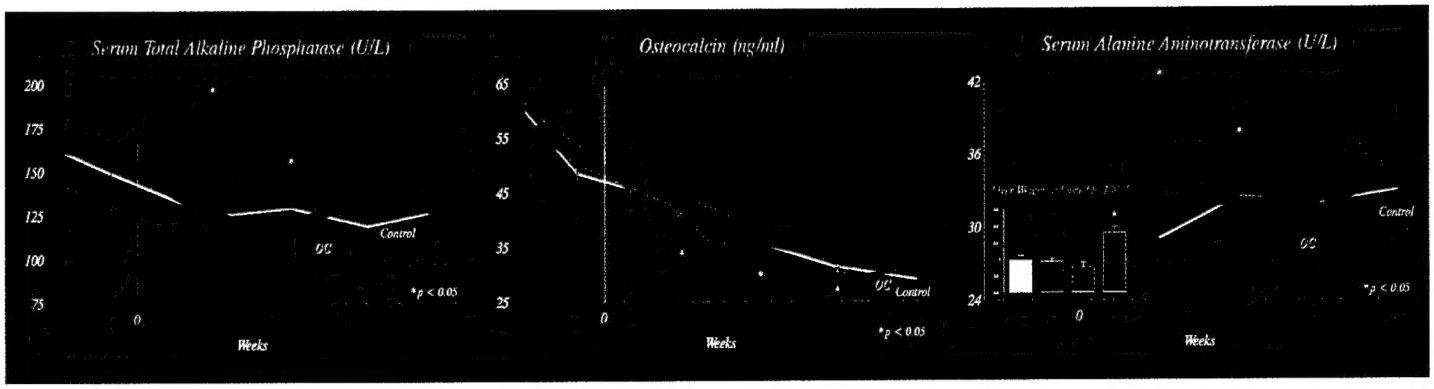
Mammary Gland-

Findings with respect to the effects of OC treatment on the mammary gland have been published (see appendix, Jayo MJ, Register TC, Hughes CL. et al. J Soc Gyn Invest7:257-265, 2000).

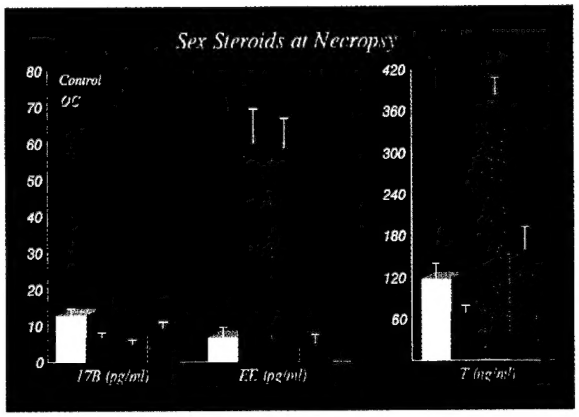
Effects of Treatments on Body weight and Bone Density Changes in body weight (BW) and lumbar spinal bone mineral density (BMDs) were observed across time. All the animals were growing before and during the experiment. All groups gained significant ($p<0.05$) BW and spinal BMD through time. *Control* and *Cas* animals gained more BW and BMD than *OC* and *OC+MT* groups ($p<0.05$).



Effects of Treatments on Bone Biomarkers Across Time Osteocalcin and ALP significantly decreased ($p<0.05$) with time in all four groups, consistent with an age dependent decline in these markers. *OC+MT* had higher levels of ALP and ALT at intermediate time points (liver effects) and lower levels of osteocalcin (bone effects) than *Control* and *OC* groups.



Effects of Treatments on Sex Steroids- At necropsy, the *OC* and *OC+MT* groups had significantly ($p<0.05$) lower serum levels of endogenous 17-beta estradiol ($p<0.05$). EE levels were significantly higher in the *OC* and *OC+MT*, and levels of T were significantly lower in *OC* and higher in *OC+MT* groups when compared to *Controls*.



Bone Histomorphometry

Method The tibial length was measured with a caliper, and the tibia was cross-sectioned one mm above the tibio-fibular junction to obtain a cortical sample. Then the bones were processed and embedded in methyl methacrylate (MMA). The proximal tibia sample and the L3 vertebrae were sectioned (at 5-10 μm) with a macrotome and mounted for 2 unstained or 2 stained (modified tetrachrome with Von Kossa method and Toludine blue with TRAP). Two sections of the cortical tibia sample were ground (approximately 25 μm), one was left unstained and the other stained with modified tetrachrome with Von Kossa. Histomorphometry was conducted using established procedure and a True Color-98 Bioquant System (R&M Biometrics, Nashville, TN). The abbreviations used are based on the ASBMR standard nomenclature.[2]

Cortical Measurements at Tibio-Fibular Junction Structural and dynamic parameters were derived separately for periosteal (Ps), cancellous (Cn), cortical (Ct), and endosteal or endocortical (Ec) bone envelopes. For each tibia, one mm above the fibular attachment, the following parameters were measured and/or derived: average cortical thickness (Ave.Ct.Th, μm), tissue volume (TV, μm^2), periosteal perimeter (Pr.P, μm), core or marrow + Cn bone volume (Core.V, μm^2), endocortical perimeter (Ec.P, μm), cancellous bone volume (Cn.BV, μm^2), cancellous bone perimeter (Cn.P, μm), hole or cortical porosity volume and perimeter (HV and HP, μm^2 and μm respectively), cortical bone volume (Ct.BV, μm^2), marrow volume (Mw.V, μm^2), percent Ct bone (Ct.BV/TV, %), percent Cn bone (Cn.BV/TV, %), periosteal single labeled surface perimeter (Ps.sLS, μm), periosteal double labeled surface perimeter (Ps.dLS, μm), periosteal inter-label distance (Ps.ILD, μm), periosteal mineralizing surface (Ps.MS/BS, %), periosteal mineral apposition rate (Ps.MAR, μm/day), periosteal bone formation rate (Ps.BFR/BS, mm^3/mm^2/day), endocortical single labeled perimeter (Ec.sLS, μm), endocortical double labeled surface (Ec.dLS, μm), endocortical inter-label distance (Ec.ILD, μm), endocortical mineralizing surface (Ec.MS/BS, %), endocortical mineral apposition rate (Ec.MAR, μm/day), and endocortical bone formation rate (Ec.BFR/BS, mm^3/mm^2/day).

Cancellous Measurements at Proximal Tibia Structural and dynamic parameters were measured and/or derived for cancellous (Cn, trabecular) bone. Although the Cn bone may be affected by growth rates, measurement was taken at a standard area of approximately 2-3 mm^2 of bone tissue at least 1.0 mm away from the growth plate to exclude the primary spongiosa. For each proximal tibia, the bone and marrow tissue volume (TV, μm^2), bone tissue area (BA, μm^2), bone surface perimeter (BS, μm), Cn bone volume (BV, μm^2), percent Cn bone (BV/TV, %), trabecular bone thickness (Tb.Th, μm), trabecular separation (μm), trabecular number (#/mm), single labeled bone surface perimeter (sLS, μm), double labeled bone surface perimeter (dLS, μm), not labeled bone surface (nL.S, μm), mean inter-label distance (MILD, μm), mineralizing surface (MS/BS, %), periosteal mineral apposition rate (Ps.MAR, μm/day), bone formation rate surface referent (BFR/BS, mm^3/mm^2/day),), bone formation rate volume referent (BFR/BV, %/yr), and bone formation rate total tissue volume referent (BFR/TV, %/yr).

Cn Measurements of L3 Vertebra Similar to the proximal tibia, structural parameters were measured and/or derived for cancellous (Cn, trabecular) bone. For each vertebrae, the bone and marrow tissue volume (TV, μm^2), bone tissue area (BA, μm^2), bone surface perimeter (BS, μm), Cn bone volume (BV, μm^2), percent Cn bone (BV/TV, %), trabecular bone thickness (Tb.Th, μm), trabecular separation (μm), and trabecular number (#/mm) were measured.

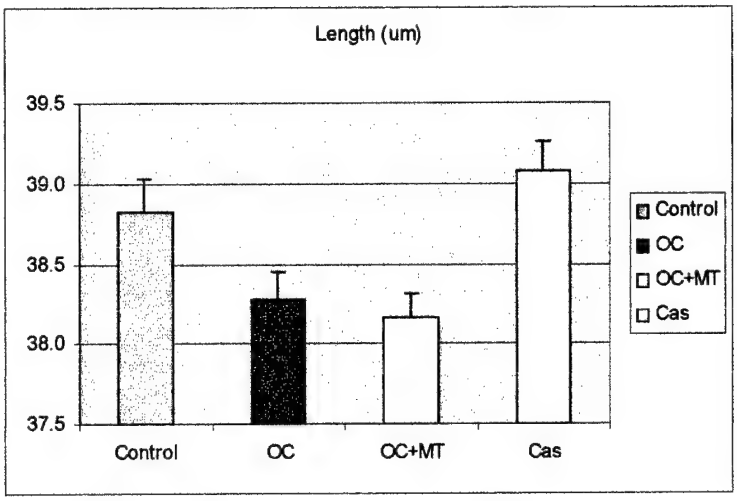
Statistics Levene's test was used to evaluate homogeneity of variances. If data was not normal and if variances were not homogeneous, non-parametric tests (Chi-square and Kruskall-Wallis tests) were used for comparisons. Normally distributed data was analyzed by Analysis of variance (ANOVA) and post hoc analyses were conducted using Tukey's honest significance test.

Results

General No significant histopathologic findings were noted. The results presented here are a synopsis of the length and histomorphometric findings.

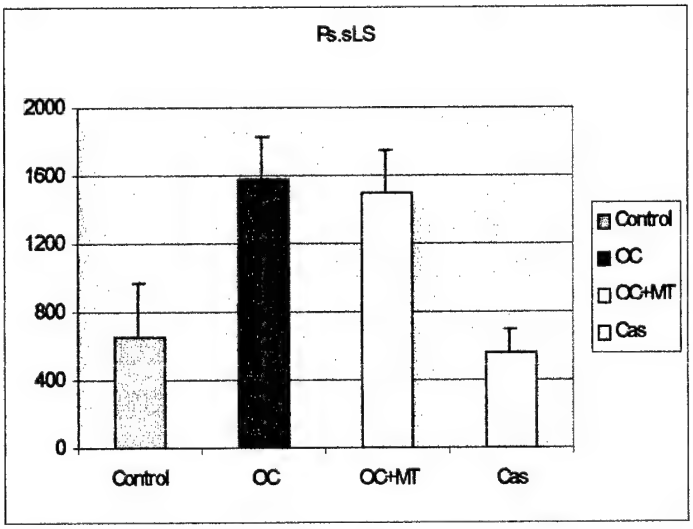
Length

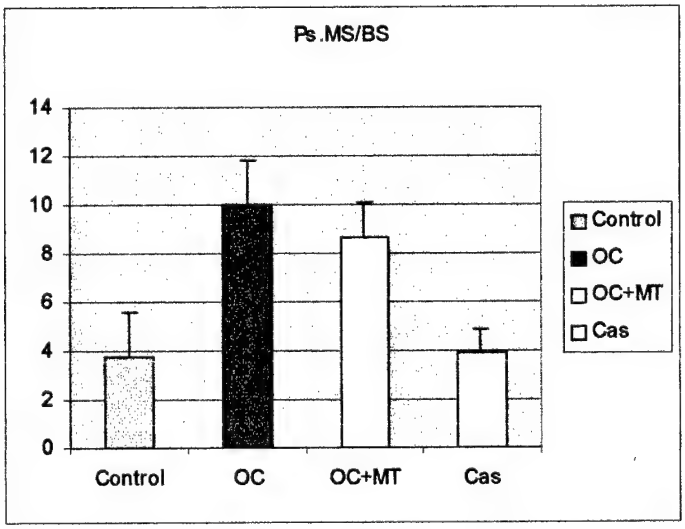
Treatment with OC's, ± MT, suppressed the longitudinal growth. By Tukey's HSD test, OC+MT treated rats had shorter tibias that Control rats ($p = 0.055$). Also, OC and OC+MT treated animals had significantly shorter tibias than the Anti-Androgen treated animals ($p^- 0.0186$ and $p^- 0.006$, respectively). OC treatment was not different than OC+MT.



Cortical Measurements at Tibio-Fibular Junction

Significant ($p<0.05$) findings were only seen for periosteal single labeled surface (Ps.sLS; $p = 0.009$) and periosteal mineralizing surface bone surface referent (Ps.MS/BS; $p = 0.011$). OC treatment ± MT caused an increase in the amount of periostal single labeled surface. Similarly, OC treatment with or without MT caused an increase in the amount of periostal single mineralizing surface.

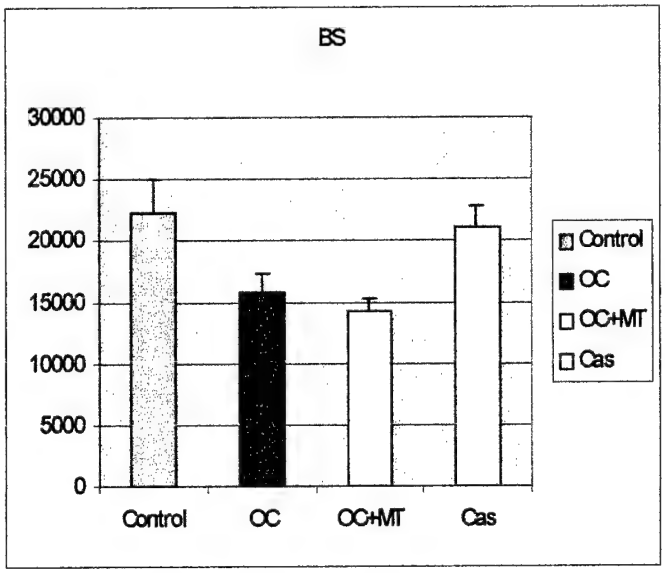


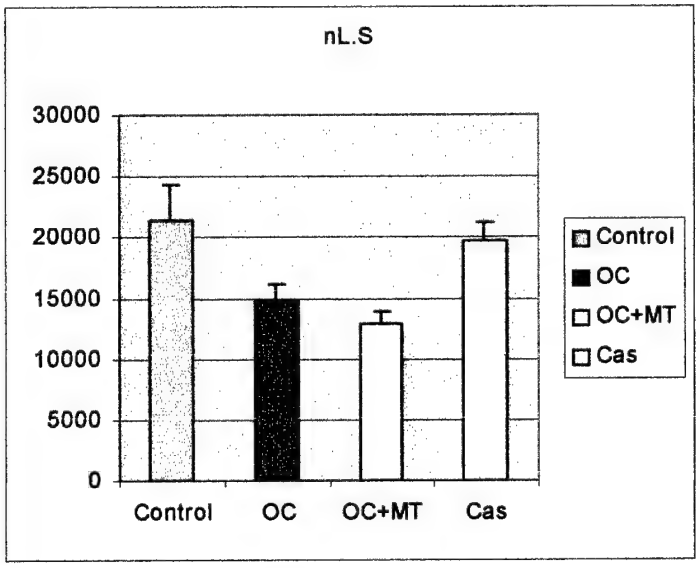


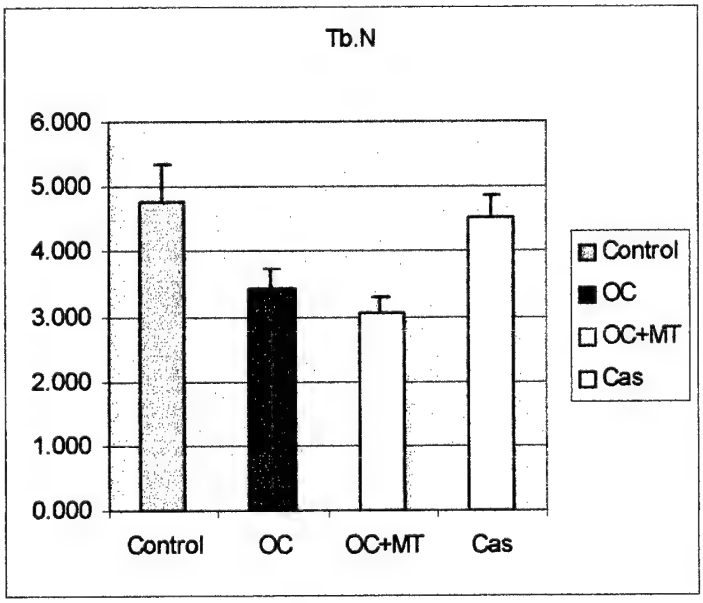
Cancellous Measurements at Proximal Tibia

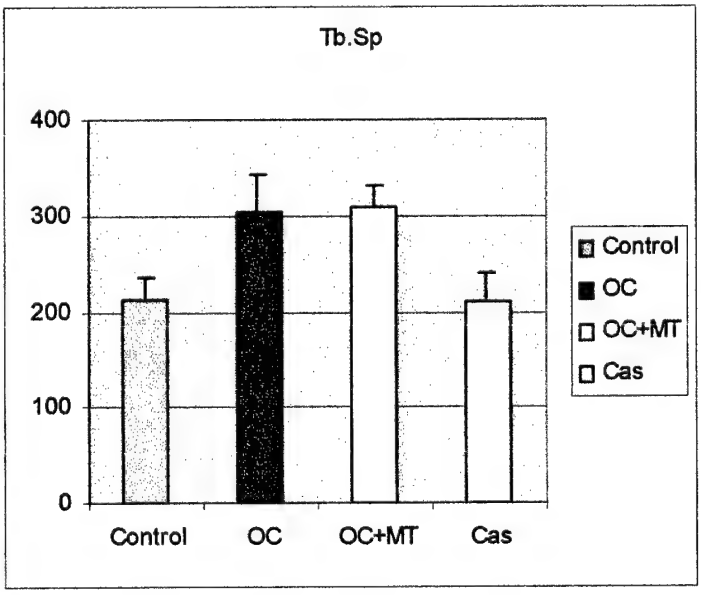
Most of the changes observed were found at the cancellous bone of the proximal tibia. The mineralizing surface bone surface referent was different ($p = 0.051$) among groups. Addition of MT to the OC treatment caused a shift in the amount of mineralizing surface. The OC group was significantly lower than the OC+MT group ($p<0.01$).

Bone surface, non-labeled surface, trabecular number, trabecular separation, bone formation rate surface referent and the amount of double labeled surface were all significantly different among the groups (ANOVA p values = 0.005, 0.005, 0.007, 0.021, 0.022, and 0.049, respectively).

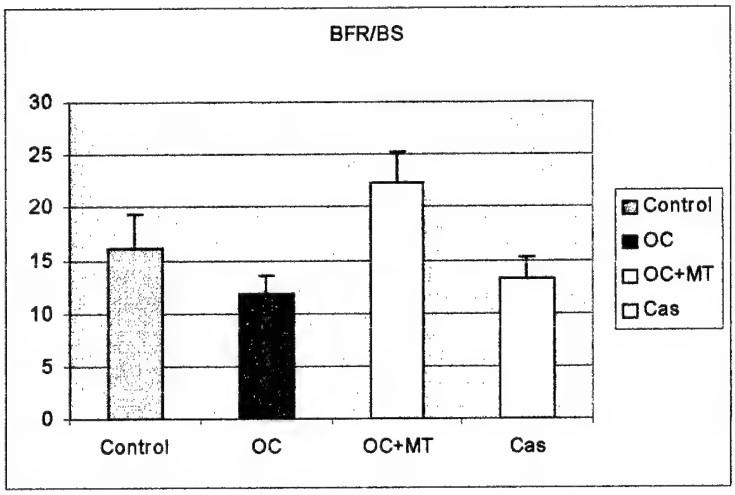


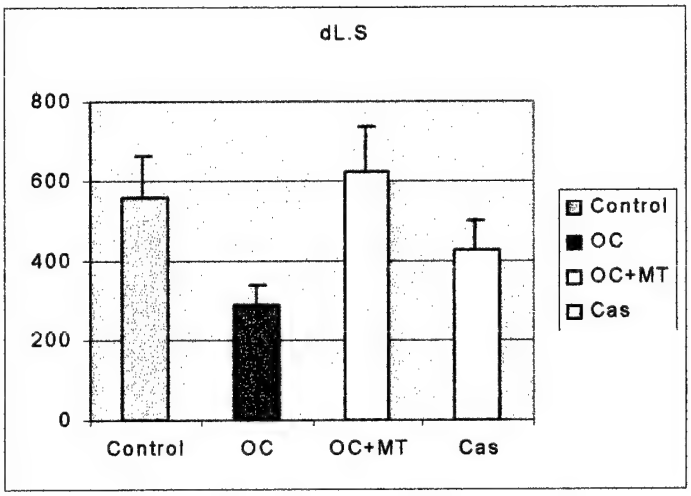






As seen in the following figures, addition of MT to the OC treatment caused a significant ($p<0.05$) increase in BFR/BS and dLS suggestive of increased bone formation. The OC+MT group was not different from the Control group.





Cn Measurements of L3 Vertebra

No significant findings were found among groups for any parameter measured.

Serum Markers of Bone Resorption (Ratlaps)

The level of whole body bone resorption can be estimated by measurement of circulating markers associated with resorption, such as degradation products of type I collagen. We assessed levels of one such degradation product (c-terminal telopeptide of the alpha chain of rat type I collagen, Serum Ratlaps, Nordic Bioscience Diagnostics, Herlev, Denmark) in serum obtained at necropsy to determine if OC treatment altered bone metabolic balance by increasing resorption. No effect of treatment was observed on Ratlaps levels ($F[3,49]=0.24$, $p=0.87$), indicating that alterations in bone resorption are not likely to be involved in the adverse effects of OC on skeletal mass.

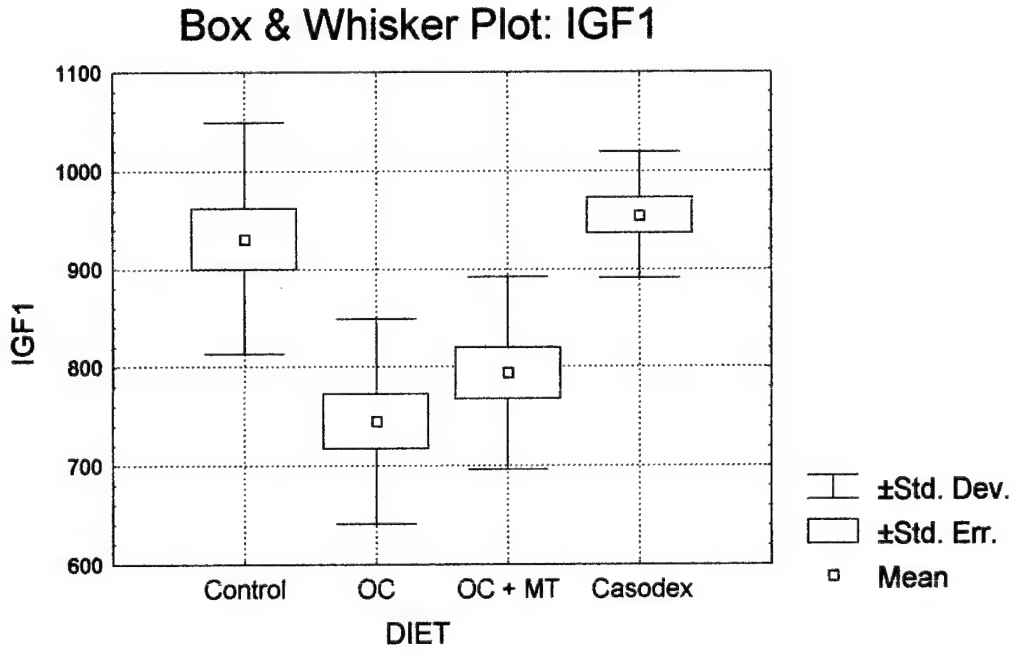
Table 8- Serum C-terminal Crosslink of Type I Collagen (Ratlaps)

	Mean	S.E.
OC	14.9	1.4
Control	15.1	1.4
OC+MT	16.5	1.7
Casodex	15.7	1.2

Effects of OC treatment on Serum Insulin-Like Growth Factor I (IGF-1)

Several lines of evidence suggest that insulin-like growth factors, in particular IGF-1, are important in the regulation of bone and cartilage growth and appear to be involved in the attainment of peak bone mass. IGF-1 production is dependent upon both growth hormone and sex hormones, although the exact relationships between these factors is not completely defined. Growth hormone deficient patients have low serum IGF-I which becomes elevated (as does bone mass) following GH replacement therapy (Bing-You et al. 1993, Jorgensen et al. 1989). Circulating IGF-I levels have been shown to be significantly correlated with bone density in post-menopausal women (Boonen 1996, Mohan 1995) . Finally, IGF-I levels in serum and the skeleton have been shown to decrease with age, as do androgens. Circulating IGF-I provide a reasonable estimate of GH secretion, which is under the regulation gonadal steroids. Thus, reductions in IGF-I may result as a consequence of OC use (perhaps via androgen supression), providing a central mechanism for inhibition of bone density increases by OC use.

I. Effects of OC on Serum IGF-1 in Sprague Dawley Rats



IGF-I concentrations were measured in serum samples obtained at necropsy using a specific assay for rat IGF-I. Samples were initially extracted with acid-alcohol to remove interfering IGF-binding proteins (DSL, Webster Tx). Results: Serum IGF-1 levels were significantly different between groups ($F[3,50]=14.3$, $p=0.00001$). Serum IGF-1 was suppressed in the OC ($p<0.0002$) and OC+MT ($p<0.003$)treatment groups relative to the Control group. This effect was not reversed by addition of MT to the OC regimen, as OC and OC+MT groups were not different from one another ($p=0.56$). Anti-androgen administration did not effect serum IGF-1 levels ($p=0.93$). These data suggested that a possible mechanism by which OC treatment inhibited bone mineral accretion involved suppression of the GH-IGF-1 axis, at least in rodents.

II. Effects of OC treatment on Serum IGF-1 in Cynomolgus Monkeys.

Two additional studies were carried out to determine if the findings in the present study regarding effects of OC treatment on serum IGF-1 and markers of bone metabolism were also observed in non-human primates. For these studies we utilized banked serum samples collected at necropsy from 2 separate completed NIH funded studies designed to examine the effects triphasic oral contraceptives on female health endpoints. Study 1, the original study which provided the rationale for this project, involved the effects of the triphasic OC containing ethinyl estradiol (EE) and levonorgestral (LN) on bone and atherosclerosis endpoints, the animals proceding on to a long term post-menopausal phase after ovariectomy at the end of Phase 1 (Register et al., 1997). Study 2 was a terminal study designed to determine the combined and individual effects of EE and LN on on the development of coronary artery atherosclerosis (Adams et al., 2000). EE and LN are the two components of the oral contraceptive regimen used in the original study by Register et al., 1997, and in the present study in the rats. In study 2, one group served as placebo-treated controls (n = 20), a second group received OC in the form of ethinyl estradiol (E2) (Wyeth-Ayerst Research, Princeton, NJ) and levonorgestrel (Wyeth-Ayerst Research) (n = 20), a third group received ethinyl E2 only (n = 21), and a fourth group received levonorgestrel only (n = 20). The hormones were mixed in the diet and animals in groups two, three, and four were treated on a 28-day triphasic schedule. The schedule was days 1 to 6, 8.2 g levonorgestrel and/or 5 g ethinyl E2; days 7 to 11, 12.5 g levonorgestrel and/or 6.7 g ethinyl E2; days 12 to 21, 21 g levonorgestrel and/or 5 g ethinyl E2; and days 22 to 28, inert placebo. As in previous experiments, menstrual cyclicity ceased in contraceptive steroid-treated monkeys. We hypothesized that serum osteocalcin levels would be suppressed by oral contraceptive treatment as previously observed by Register et al. 1997 and that serum IGF-1 levels would be suppressed as in the present study in rats. Suppression of serum IGF1 in both studies would provide support for the idea that suppression of central hypothalamic- growth hormone- IGF1 axis could be a major mechanism by which bone metabolism would be impaired by OCs.

Results and Conclusions

IGF-I concentrations were measured in serum samples obtained at necropsy using an assay for human IGF-I which had been previously validated for use in monkeys. Samples were initially extracted with acid-alcohol to remove interfering IGF-binding proteins (DSL, Webster Tx). Our initial data from Study 2 did not confirm our previous findings in the rat with respect to effects of OC on serum IGF-1 concentrations, in fact suggesting that IGF-1 levels were elevated by OC treatment in the monkeys. Subsequent reexamination of the circumstances surrounding collection of serum at necropsy revealed that the individual experimental groups were handled differently at necropsy. We carried out additional testing of serum obtained from timepoints prior to necropsy, when all conditions were equivalent, in order to verify the effects of these treatments on serum IGF1. Reanalysis of serum from a separate timepoint confirmed the data with respect to IGF-1. We also evaluated serum collected from animals after 20 months of OC treatment in Study 1, and found that OC treatment caused a significant elevation of serum IGF-1 relative to controls (IGF-1 408 +/- 19 ng/ml vs 165 +/- 13 ng/ml respectively, $F[1,186]=111$, $p<0.0001$). Taken together, these data suggest that the inhibitory effects of OC treatment in monkeys were not mediated through suppression of serum IGF-1.

Effects of OC treatment on serum estradiol concentrations in female monkeys

An alternative mechanism by which OC might inhibit increases in bone mineral content and density during development is via suppression of serum estrogen concentrations. We assessed serum estradiol concentration in OC treated and Control cynomolgus monkeys after 20 months of treatment (the same samples used for serum IGF-1 analyses in Study 1, see above). As expected, OC treatment resulted in a significant reduction in serum estradiol levels relative to controls (35.8 +/- 3.2 pg/ml in the OC group vs. 63.1 +/- 5.5 pg/ml in Controls,

F[1,179]=19.6, p<0.0001). These data provide support for an alternative mechanism by which OC treatment may impair increases in skeletal mass during young adulthood. Additional support for this hypothesis comes from the findings in the non-human primate females in Study 1, as the OC-induced deficits in bone mass relative to controls were recovered when the animals were ovariectomized and treated with conjugated equine estrogens (Jayo et al.1998). In other words, control (untreated) animals in the pre-menopausal phase of the study maintained their bone mass following ovariectomy and treatment with CEE, while animals treated with OC during the premenopausal phase increased their bone mass following ovariectomy and CEE treatment to the level of the phase I controls treated with CEE in phase II.

KEY RESEARCH ACCOMPLISHMENTS

The key findings of the study are:

1) OC use decreased the peak bone mass of young intact female rats, similar to the findings in cynomolgus monkeys.

2) Addition of a non-aromatizable androgenic steroid to OCs, at the dose provided, did not prevent the adverse effects of OCs to the growing skeleton of young rats.

3) Anti-androgen treatment did not cause an adverse effect on the growing skeleton of young rats at the achieved dose, contrary to the hypothesized effects.

4) Histomorphometric data suggest that OC use suppressed bone turnover, however these findings were not reflected in biomarkers of bone formation or resorption..

5) Addition of MT to OC appeared to increase bone formation rate determined histomorphometrically, but this was not reflected in biomarkers of bone metabolism.

6) Unlike the effects in monkeys, OC use did not reduce biomarkers of bone formation (osteocalcin and alkaline phosphatase) in the rats.

7) Effects of OC on bone density were paralleled by alterations in serum IGF-1 in the rats. However, female monkeys treated with OCs had *increased* serum IGF-1, suggesting that suppression of IGF-1 is not the mechanism through which OCs inhibit bone.

8) OCs decreased serum estradiol concentrations in rodents and monkeys, providing support for an alternative mechanism for the adverse effects of OC on bone mass as estrogen is a potent bone promoting/protective hormone.

9) Species specific biologic responses (e.g. serum osteocalcin, B-ALP, IGF-1) of the rat to OC treatment may limit its utility as an animal model for the study of OC effects.

REPORTABLE OUTCOMES

Abstract presented at the 1999 Meeting of the American Society of Bone and Mineral Research
Jayo MJ, Register TC, Hughes CL, Blas-Machado U, Sulistiawati E, Louderback PW, Rankin SE. Oral contraceptives and androgens: Effects on bone mass acquisition in female rats. J Bone Miner Res 1999; 14(Suppl 1):

Abstract presented at the 2001 Meeting of the Endocrine Society
Register, TC and Jayo, MJ. Oral contraceptive inhibition of bone growth in young female rats: the role of androgens. Proceedings of the 83rd Annual Meeting of the Endocrine Society, P2-361, June 2001.

Abstract presented at the 2001 American Society for Bone and Mineral Research Meeting
Reference: Register, TC and Jayo, MJ. Oral contraceptive inhibition of bone growth in young female rats: the role of androgens. J Bone Miner Res 2001;16(Suppl 1):S317.

Manuscript published in the Journal of the Society for Gynecologic Investigation
Jayo MJ, Register TC, Hughes CL, Blas-Machado U, Sulistiawati E, Borgerink H, Johnson CS. *Effects of an oral contraceptive combination with or without androgen on mammary tissues: a study in rats.* J Soc Gynecol Investig 2000 Jul-Aug;7(4):257-65

Tissue, serum, and urine repository- We have banked and extensive collection of organs, tissues, serum, urine, and biological extracts from this study which are being used to further the scientific endeavor through emerging technologies including DNA arrays with a potential for proteomic applications.

An extensive database of all results are maintained as a part of the continuing research effort.

Research and funding opportunities have developed with the private sector as a result of this research.

Two or more additional manuscripts will be published from these results.

CONCLUSIONS

The results from this project suggest that OCs may inhibit bone metabolism and the acquisition of peak bone mass in rats, in part confirming the previous finding in cynomolgus macaques (Register et al., 1997). The addition of a non-aromatizable androgen (MT) to the OC did not significantly counteract the effects of OC treatment on bone mass and the anti-androgen bicalutamide did not mimic the effects of OC treatment on bone density or metabolism, suggesting that hypoandrogenemia does not play a central role in the effects of OC on bone in young adult females. Androgens, natural or synthetic, are not part of any OC therapy available to women, and to our knowledge, this is the first time that the effects of addition of androgens to estradiol containing OCs on bone tissues of skeletally immature and reproductively sound subjects have been evaluated.

The histomorphometric data demonstrated that OC treatment with or without MT caused significant osteopenia at the proximal tibia cancellous bone, with increased tabecular separation and a decrease in the trabecular number. In addition, double labeled surface and the BFR/TV were significantly ($p<0.03$) lower in the OC-treated animals compared to Controls. These findings suggest a mild suppression of bone formation/turnover in the OC treated animals compared to Controls. Species specific responses to OC treatments were also observed. In this study OC did not alter circulating markers of bone formation ALP and

osteocalcin, although both were significantly suppressed in OC-treated monkeys. It is possible that changes in circulating osteocalcin observed with age in the rat study overwhelmed any suppressive effect of OC treatment. Peak circulating levels of osteocalcin in the rat are found at about 21 days of age and rapidly and significantly decrease to a nadir by 16 weeks of age (Liu and Lin). Alternatively, this could be indicative of a dose effect since the rats received 30% less than the non-human primates based on the human dose. We also found that OC treatment suppressed serum IGF-1 in the rats while elevating IGF-1 in the monkeys, suggesting that systemic IGF-1 is not the key mediator of the OC inhibition of bone mass. It is possible that reductions in serum IGF-1 in the rats are related to reduced diet consumption.

MT supplementation to OC treatment had no effect on BMC or BMD relative to OC treatment alone, although certain bone metabolic and histomorphometric parameters were effected. For example, addition to OCs of MT significantly suppressed osteocalcin levels relative to the OC group. Histomorphometrically, BFR/BS, MS/BS, dL.S, and BFR/BV were all significantly higher (<0.03) in the OC+MT group compared to the OC only treated animals. It is not known if dose modification would provide beneficial effects to the skeleton, although it is worth noting that addition of MT to OC also caused liver effects as demonstrated by elevations of serum ALT. These serum markers changes were not apparent in the liver grossly (see liver weight bar graph) or histologically (not presented).

Young women who take OCs suppress endogenous formation and serum levels of bioavailable sex hormones, by direct and central negative feedback and by indirectly affecting the circulating levels of SHBG. We found that serum concentrations of androgens and estradiol were reduced by OC treatment in both the rat and monkey. Consequently, the level of bioavailable androgen and estrogen at the tissue level are modulated by OCs. Estradiol is a potent bone protective hormone which is critical for normal growth and development of the skeleton. Effects at other hormone sensitive organs (endometrium and mammary gland) are also likely. Taken together, these studies suggest that the suppression of estrogen levels rather than androgens may be responsible for the inhibitory effects of OC on bone mass accrual in females prior to the attainment of peak bone mass. Additional studies are required for verification of this hypothesis.

Confounds to the present study

The results obtained in this study, at least as far at the effects of OC treatment, are somewhat confounded by the failure of the rats to consume their diets containing the OC. The differences in diet consumption led to differences in body weight, which is generally associated with bone mass and density. Such alterations in diet consumption were not observed in the previous study (Register et al., 1997) in cynomolgus monkeys which served as the stimulus for this project. Nevertheless, the addition of the non-aromatizable androgen to OC treatment did not affect diet consumption relative to the OC only group, neither did the addition of the androgen antagonist relative to the control group receiving no hormone therapy. The finding that the rats in this study did not eat equivalently the diets containing the hormones has some precedent, despite our pilot studies which suggested otherwise. Manoharan, et al (1970) used diet as the method for OC delivery which led to less food consumption and lower BW. Interestingly, SQ injections of OC also have led to reductions in BW (Lea et al., 1996). Regardless as to cause, lack of appetite and/or food aversion, BW were significantly reduced in the OC and OC+MT groups. It is difficult to determine the absolute role that alterations in BW play in the effects of the OC and OC+MT treatment on the skeleton. BW at scan time 12 weeks correlated positively and significantly ($p<0.05$) with spine BMD and pQCT TBMD at the same time point ($r=0.566$ and 0.429, respectively). However, the amount of diet and drug consumed was sufficient to provide for measurable differences in circulating sex hormones, and liver and bone biomarkers.

In summary, although interpretation is complicated by the BW effects, our findings support the previous finding that OC use by young individuals appears to prevent proper bone accrual and maximal peak bone mass (PBM) (5-7). OCs, at the dose and route given, negatively affected acquisition of PBM and skeletal integrity in young rats. Supplementation of OCs with androgens, in the dose and form of MT, failed to prevent the OC-induced bone effect. Use of the anti-androgen Casodex®, at the dose provided, did not cause adverse skeletal effects. Overall the data do not support a role for hypoandrogenemia in the inhibitory effects of OC on bone mineral acquistition in young adult animals. In effect, androgen supplementation may not be adequate to prevent adverse effects of OC on the skeleton. Initial findings regarding the reduction of serum IGF-1 levels suggest an inhibition of GH secretion via the HPA axis may underly some of the OC effects. Additional studies into the mechanism by which OCs influence normal bone acquisition are necessary before full understanding of the effects are achieved. However, it is possible that it is the ultimately the suppression of estrogen levels rather than androgens that is responsible for our observations of OC inhibition of bone mass accrual in females prior to the attainment of peak bone mass.

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APPENDICES

A. Reprint of the following publication:
Jayo MJ, Register TC, Hughes CL, Blas-Machado U, Sulistiawati E, Borgerink H, Johnson CS. *Effects of an oral contraceptive combination with or without androgen on mammary tissues: a study in rats.* J Soc Gynecol Investig 2000 Jul-Aug;7(4):257-65

Effects of an Oral Contraceptive Combination With or Without Androgen on Mammary Tissues: A Study in Rats

Manuel J. Jayo, DVM, PhD, Thomas C. Register, PhD, Claude L. Hughes, MD, PhD, Uriel Blas-Machado, DVM, PhD, Erni Sulistiawati, DVM, Hermina Borgerink, BA, and Christopher S. Johnson, BS, MS

OBJECTIVES: *Oral contraceptive (OC) therapy has long been known to produce hypoandrogenemia. However, androgens are not part of any OC therapy available to women. This project was designed to evaluate the effects of low-estradiol containing OC, with or without methyltestosterone (MT), on cell proliferation and progesterone receptor (PgR) expression in mammary gland epithelia of virgin female rats.*

METHODS: *Sixty rats were divided into four groups. One group received OCs, whereas a second group received OC plus MT. A third group of rats was treated with an antiandrogen to mimic the hypoandrogenemic effects caused by OC therapy. All treated groups were compared with age-matched untreated controls.*

RESULTS: *After 15 weeks of treatment, no inflammatory, precancerous, or cancerous lesions were observed in any treatment group. OC plus MT therapy caused significant suppression of epithelial proliferation, a reduction in the number of proliferating cell nuclear antigen–labeled cells, and an increase in the number of PgR-labeled cells.*

CONCLUSIONS: *Our results suggest that a medication containing an estrogen–progestin–androgen combination has antiproliferative effects in mammary glands of experimental animals that could prove to have breast-protective potential in women. (J Soc Gynecol Investig 2000;7:257–65)* Copyright © 2000 by the Society for Gynecologic Investigation.

KEY WORDS: Mammary glands, oral contraceptives, androgens, cell proliferation, progesterone receptor.

Women who develop breast cancer in their premenopausal years tend to have subnormal serum levels of adrenal androgens. Consequently, it has been proposed that androgens, possibly by acting through the androgen receptor (AR), may oppose estrogen-stimulated cell growth in premenopausal years.[1] Epidemiology, genetics, in vitro work, and anticancer therapy support the hypothesis that circulating androgens protect against breast cancer risk. Because breast cancer in men is rare and affects <0.1% of the male population, gender incidence supports a beneficial effect of androgens. Epidemiologically, retrospective case-control studies indicate that both estrogen excess and androgen deficiency may be involved in male breast cancer. In fact, the strongest association between aberrant endocrine function and male breast cancer occurs in patients with Klinefelter's syndrome, who have an approximate 3% lifetime risk of developing breast cancer.[2] Also, heritable genetic mutations in the AR gene are associated with a predisposition to male breast cancer development.[3] These findings are supported by in vitro molecular studies on tumor cells, which provide evidence for a beneficial effect of androgens. The proliferation of AR-positive malignant mammary cell lines (MFM-223) can be inhibited with androgen treatment.[4] Finally, anticancer therapy supports androgen's beneficial action, because for years androgens such as synthetic methyltestosterone (MT) have been part of the chemotherapy armamentarium available to clinicians treating women with breast cancer.

Oral contraceptive (OC) therapy in women creates an additional challenge in the understanding of breast cancer risk and development. Women may take contraceptives for a long time, and the role of exogenous estrogens, such as those present in OCs, as risk factors for developing breast cancer remains controversial. OC treatment even at a low dose causes a significant decrease in androgen. In fact, OCs are commonly

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Supported in part by grant no. DAMD117-98-1-8514 of the U.S. Department of Defense and a grant from Watson Pharmaceuticals.

This project would have been impossible without the technical assistance of Sam Rankin, BA, Pam Louderback, BS, Dianne Wood, Kyrum Martin, and Jean Gardin, BS. The authors thank J. Mark Cline, DVM, PhD, and Shannon Mathes, BS, MBA, for technical advice. Some of the concepts presented here are part of U.S. Patents 5,770,226 and 5,962,021, which are properties of Wake Forest University Medical School.

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PII S1071-5576(00)00059-9

Table 1. Semipurified Diet

Food	g
Casein, USP	10.5
Lactalbumin	10.0
Dextrin	30.6
Sucrose	28.0
Alphacel	10.0
Lard	5.20
Safflower oil (linoleic)	1.00
Choline bitartrate	0.20
Vitamin mixture, AIN-76A	1.00
Mineral mix, AIN-76	3.50

Each 100 g of semipurified high-fat diet contained the products listed.

used to treat hirsutism and to correct hyperandrogenemic states in women.[5–9] Another well-documented effect of OC therapy is a significant increase (>150%) in sex hormone-binding globulin (SHBG) levels.[7,10–12] Thus, addition of an androgen such as MT to an OC formulation should both decrease the circulating levels of SHBG and provide androgens to compensate for the diminished androgen status of women receiving OC therapy who have subnormal or lower limits of normal levels of androgens.[13–15]

In summary, the endogenous formation and serum levels of bioavailable sex hormones in women taking OCs are suppressed directly by central negative feedback and indirectly by affecting the levels of SHBG. The net effect that all of these endocrinologic changes may have on the mammary gland is unknown and is probably difficult to evaluate completely in women. Because androgens, natural and synthetic, are not part of any OC therapy available to women, this project was originally designed to evaluate the effects of low-estradiol containing OCs, with or without androgens, on bone tissues of skeletally immature and reproductively sound rats.[16] However, in this publication we focus on the effects that these hormone combinations have on the proliferation (by proliferating nuclear antigen [PCNA]) and progesterone receptor (PgR) expression of epithelia in the mammary glands of young female rats.

MATERIALS AND METHODS

Animals

Sixty specific pathogen-free virgin female Sprague–Dawley rats aged 40 days old were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN) and transported to the animal facilities of the Section on Comparative Medicine at the Wake Forest University School of Medicine (WFUSM). Guidelines established by WFSUM's Institutional Animal Care and Use Committee, state and federal laws, and standards of the Department of Health and Human Services were followed throughout the experiment. The rats were provided water ad libitum by automatic watering systems. Animals were fed a drug-free, high-fat, semipurified diet composed of (as a percentage of calories) 19.5% protein, 15.9% fat, and 64.6% carbohydrates. Diets were devoid of isoflavonoids (Table 1). During the baseline period, the rats were fed this diet, weighed daily, and acclimated to the animal facilities at WFUSM. The

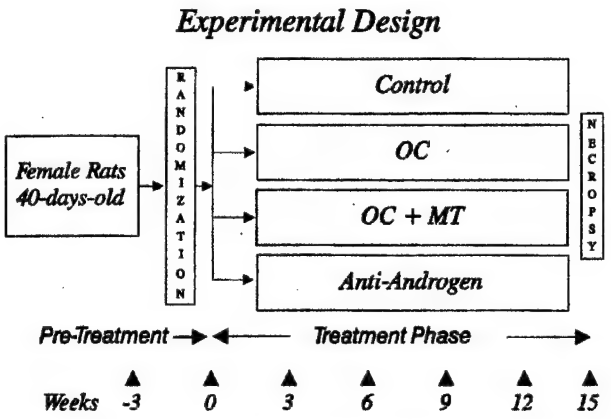

Figure 1. Flow chart of the experimental design. Sixty 40-day-old virgin female rats were randomized into four treatment groups. Treatment was started on day 70 and continued for 15 weeks. Treatment groups consisted of control, OC, OC+MT, and antiandrogen (bicalutamide). OC- and OC+MT-treated animals were treated for 3 days on and 1 day off; antiandrogen treatment was daily.

rats were housed singly (20 × 32 × 20-cm cages) and kept under constant environment (24C and 12:12 h light/dark cycle) throughout the experiment. Each rat received 25 g of food per day, and every morning their food intake (measured indirectly from leftover food) and body weight (BW) were recorded. During the pretreatment period, the rats were assessed with daily vaginal cytology for five consecutive days each week. All of the rats showed three continuous estrous cycles of 4 to 5 days of duration.[17]

Characteristics of Animal Model Used

We selected rats because they are the most extensively studied mammalian model for the effects of ovarian function and hormonal therapy on bone.[18,19] In addition, the rat has been shown to be a good model for studying the role of hormones in breast cancer because young rats, like parous women, become nearly refractory to mammary carcinogenesis after delivering offspring. Finally, recent data in the virgin rat model suggest that estrogen and progesterone therapy, besides being nontoxic and at doses that mimic pregnancy, may protect against mammary cancer.[20] According to Moore,[17] puberty in rats is reached at age 50–60 days, with the minimum breeding age required being 55–90 days (approximately 250 g). We selected the age to start treatment (70 days) because the animals are adults based on reproductive capacity at this age. On the other hand, bone studies have shown that 70-day-old rats have significantly reduced longitudinal growth[21] but have not yet reached peak bone mass.[22,23] Consequently, initiation of treatment at 70 days provided for OC therapy during a period in a rat's life that theoretically coincides with the human adolescence.[24]

Experimental Groups, Oral (Dietary) Treatment Regimen

Figure 1 shows that 40-day-old rats were randomized, based on BW, into four groups of 15 rats each. The rats were

monitored and given the semipurified baseline diet (described previously) (Table 1) for 3 weeks before treatment. At 70 days of age (approximately 227 g), the groups were treated, with or without treatments mixed in their diet, for 15 weeks as follows: 1) control, 2) OC (Levlen; levonorgestrel [LNG] + ethinyl estradiol [EE] at 0.0310 mg and 0.00619 mg per 100 g of diet, respectively), 3) OC + MT (OC as in group 2 + Android [ICN Pharmaceuticals, MT at 0.516 mg/100 g]), and 4) antiandrogen (Casodex or bicalutamide; Zeneca Pharmaceuticals, an antiandrogen at 10.33 mg/100 g). Doses were prepared to mimic a woman's contraception (0.5 mg LNG + 0.03 mg EE), postmenopausal hormone replacement (2.5 mg MT), and a man's anticancer (50 mg bicalutamide) daily dose based on 1800 cal/day. The calculations for the amount of diet to be given per rat were based on the fact that rats eat an average of approximately 32 calories/100 g of BW per day (for example, a 195-g rat will receive about 25 g diet/day). The diets were prepared every 2 weeks and kept frozen. After thawing for first use, diets were kept in the dark and refrigerated until ready for reuse. Control animals received the base diet every day. OC and OC+MT groups received steroid-containing diets for 3 consecutive days and base diet on the fourth day (similar to a rat's estrous cycle and proportional to the 3 weeks out of 4 in OC therapy cycles for women). Animals in the antiandrogen group received the antiandrogen-containing diet every day throughout the experiment. Both food consumption and BW were measured daily to monitor health status and drug intake.

Serum Steroids

Serum was collected at the time of necropsy for determination of endogenous 17β-estradiol (E2), exogenous ethinyl 17α-estradiol (EE), and testosterone (T) using commercial radioimmunoassay kits according to the manufacturer's guidelines (Diagnostic System Laboratories [DSL], Webster, TX). Serum collections were random (not scheduled for cycle stage) for control and antiandrogen groups. Animals in the OC and OC+MT groups were sampled on the second or third days of the contraceptive pill 4-day cycle. Briefly, serum estradiol was measured using the Ultrasensitive Estradiol assay (DSL-4800), which has a theoretical lower limit of detection of 2.2 pg/mL and a low standard concentration of 5 pg/mL. For the studies reported here, all E2 values < 2.2 pg/mL were arbitrarily set to 2.1 pg/mL. There was no difference in statistical outcomes whether E2 values < 2.2 were used as calculated from the radioimmunoassay (RIA) standard curve or arbitrarily set to 0 or 2.1 pg/mL. Serum EE levels were measured using DSL-9500, which has a low standard of 10 pg/mL. EE values < 10 were set to 9.9 pg/mL. Serum testosterone was measured using DSL-4100, which has a theoretical lower limit of 50 pg/mL and a low standard of 100 pg/mL. The cross-reactivity of the RIA is 6.6% with 5α-dihydrotestosterone (DHT) and undetermined for MT. No T values were less than the 50 pg/mL lower limit.

Tissue Collection at Necropsy

At necropsy, each rat was deeply sedated and a final in vivo blood sample (through the jugular vein) was collected. The rats were then euthanized by intraperitoneal injection of pentobarbital (200 mg/kg) and exsanguinated intracardially. The hair was clipped off the abdomen. The left caudal abdominal and inguinal mammary glands (consisting of epithelial glandular tissue, connective tissue, and the covering skin) were dissected together and laid flat on filter paper. These tissues were immersion fixed in neutral-buffered 10% formalin for no more that 48 hours to optimize preservation of antigenic sites, followed by processing through graded alcohols, clearing through xylene, and embedding in paraffin.

Histology and Immunohistochemistry

Procedures used were provided in part in the data sheet given with each primary antibody used and from published procedures.[25,26] Briefly, serial sections were taken from each block, picked up on regular Plus (Surgipath, Richmond, IL) as well as ProbeOn Plus (Fisher, Raleigh, NC) slides from a protein-free waterbath. Slides were designated for routine hematoxylin & eosin (H&E) staining, as well as for PCNA and PgR immunohistochemical localization. Slides designated for immunostaining were air-dried overnight and stored in a dust-free plastic slide box until ready for use. After deparaffinization, the slides were placed in distilled water. To produce heat-induced antigenic retrieval, citrate buffer at pH 6.0 was heated in vented coplin jars for 4 minutes at high power in a microwave oven.[27] The slides were then immersed into the hot buffer and allowed to sit covered for 20 minutes. After washing in running deionized water (5 minutes), the slides were placed in Tris wash buffer containing 0.5% casein (to block nonspecific binding) and 0.001% Triton X-100 for 5 minutes (to minimize surface tension and improve capillary action). Paired slides were then loaded in a ProbeOn (Fisher) handheld slideholder, including the appropriate negative control slides. Negative controls did not receive primary antibody but instead received mouse normal immunoglobulin Gs (IgGs). The epithelium of the stratum basale served as positive control for PCNA-immunostained slides. The nipple and its ductular epithelium served as the positive controls for PgR-immunostained slides. All test slides received primary antibodies which were incubated overnight at 4C.

Progesterone receptor–immunostained slides received PR10A9 as the primary antibody (IgG2a; BioGenex, San Ramon, CA) at a dilution of 1:1000. We then detected PgR-positive nuclei using an avidin–biotin horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) and DAB (HK153-5K, Liquid DAO Substrate Pack; BioGenex, San Ramon, CA) as substrate. This staining required blocking of endogenous peroxidase activity with 3% H_2O_2 before primary antibody incubation at room temperature. The slides were washed in running deionized water for 5 minutes and stained with Mayer's hematoxylin. After washing, dehydration though graded alcohols, and clearing through

Figure 2. Sample photomicrographs of subjective histologic scores. A) Score = 0; minimal alveolar proliferation primarily ducts are seen without alveolar formation. B) Score = 1; mild alveolar proliferation seen around ducts. C) Score = 2; moderate alveolar proliferation in which ducts become less prominent owing to alveolar development. D) Score = 3; marked alveolar proliferation around ductules with lobule formation. H&E stain, original magnification ×25.

xylenes, all slides were immediately coverslipped with permount (Figure 2).

The PCNA-immunostained slides received PC10 as the primary antibody (IgG2a; Novocastra Laboratories Ltd., distributed by Vector Laboratories) at a dilution of 1:100 for 20 minutes. As shown in Figure 2, PCNA-positive nuclei were then detected using a streptavidin-alkaline phosphatase system with an alkaline phosphatase substrate kit (Vector Red Substrate kit, SK-5100; Vector Laboratories).

Histopathology and Cell Counts

Histopathologic evaluations were conducted by veterinary pathologists and each H&E section was evaluated subjectively and graded from 0 to 3 (low to high) based on alveolar proliferation (Figure 3). Morphometric histology was developed and modified from previous publications.[25,26] For cell counts, a 20 × 20 grid was randomly placed over a microscopic field of subcutaneous skin, adjacent to mammary gland tissue, creating a field area of 0.24 mm^2. The epithelium covering the teat canal or major ducts was not included in the cell count. Each slide was moved in a right up–right down–right up sequence to avoid counting previously measured areas, and without counting the left and top edge intercepts (limiting intercepts to a total of 400 per grid area). The total number of glandular epithelial cell nuclei touching a grid line intercept was counted until a total of 100 cells were counted. However, when a field was started, it was completely counted even when more than 100 total nuclei were counted.

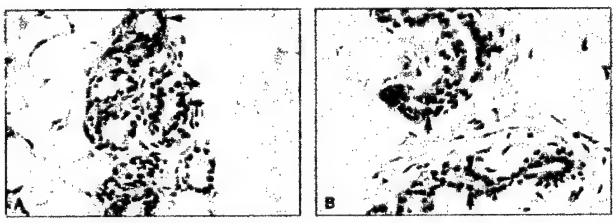

Figure 3. Sample photomicrographs of immunohistochemical histology slides. A) PgR-positive nuclei stain dark to golden brown (*arrowheads*). B) PCNA-positive nuclei stain red (*arrowheads*). Original magnification ×50.

For each PCNA- and PgR-immunostained slide, the following values were measured and/or derived: 1) number of microscopic fields necessary to count at least 100 nuclei touching grid line intersection at ×20 magnification (field#); 2) total area measured (area, as the field# × conversion factor of 0.24 mm^2); 3) total number of all nuclei, regardless of staining, and touching a grid line intersection (total nuclei); 4) number of unstained nuclei at a grid line intersection ([−]nuclei); 5) number of stained nuclei at a grid line intersection ([+]nuclei); 6) percentage of unstained nuclei (%[−], as unstained nuclei/total nuclei × 100); 7) percentage of stained nuclei (%[+], as stained nuclei/total nuclei × 100); 8) total number of nuclei per area (nuclei/mm^2); 9) number of unstained nuclei per unit area ([−]/mm^2); and 10) number of stained nuclei per unit area ([+]/mm^2). One PCNA-labeled slide was counted five times, with stage replacement, to calculate measurement variability. Based on this slides, the coefficient of variation was <3%.

Statistics

We conducted all statistical calculations using a statistical software package (Statistica; StatSoft, Tulsa, OK). For mammary gland parameters, statistical analyses contained 14 of the 15 animals in the control, OC, and OC+MT groups and 12 of the 15 animals for the antiandrogen group. Mean and standard errors of the mean (SEMs) were calculated and presented in all tables and figures. Analysis of variance and post-hoc Tukey comparison tests were conducted for each measurement. Levene's test was used to test for unequal variances, and nonparametric data were tested by median (chi-square) statistics. Sex steroids post hoc comparisons between the control and treatment groups were made using the Bonferroni method.

RESULTS

BW and Food Intake

The groups had equivalent BW at baseline and all groups gained significant BW through time ($p < .05$). However, at necropsy the animals in the control and antiandrogen groups were significantly heavier than in the OC or OC+MT ($p < .001$). In this study all groups ate less food than predicted, based on BW. As seen in Table 2 and based on daily dietary consumption records, the animals in the control group did not eat more than 25 g of food per day, regardless of BW. On average (the mean intake of a 4-day cycle), the groups ate approximately the same amount of food as a percentage of their BW. As shown in Figure 4, the groups differed in dietary intake by day of the pill cycle. The OC and OC+MT groups ate significantly less ($p < .05$) than the control and antiandrogen groups on the first or second day of the pill cycle, approximately the same percentage of diet by the third day, but

Table 2. Body Weight, Food Intake, and Food Intake as a Percentage of BW (Food/BW%) Measured Across the 4-Day Pill Cycle and Averaged for the Experiment (Mean ± SEM)

	Control	OC	OC+MT	Antiandrogen
BW (g)	272.46 ± 0.49	233.53 ± 1.46	231.42 ± 1.54	269.49 ± 0.36
Food (g/day)	20.02 ± 0.17	16.79 ± 2.57	17.09 ± 2.66	19.35 ± 0.26
Food/BW %	7.35 ± 0.05	7.17 ± 1.04	7.36 ± 1.09	7.18 ± 0.09
Expected LNG intake		2.67*	2.67*	
Actual LNG intake		1.91 ± 0.12	1.97 ± 0.18	
Expected EE intake		0.533†	0.533†	
Actual EE intake		0.38 ± 0.02	0.39 ± 0.04	
Expected MT intake			44.44‡	
Actual MT intake			32.80 ± 3.04	
Expected antiandrogen intake				890§
Actual antiandrogen intake				750 ± 10

Dosing is provided as expected and actual intakes for levonorgestrel, ethinyl estradiol, and methyltestosterone and for Casodex (antiandrogen) which are expressed as μg/100 g BW per day.

* To mimic a human dose of 0.15 mg LNG/1800 calories per day.

† To mimic a human dose of 0.03 mg of EE/1800 calories per day.

‡ To mimic a human dose of 2.5 mg MT/1800 calories per day.

§ To mimic a human dose of 50 mg/1800 calories per day.

significantly more diet ($p < .05$) than the control or antiandrogen groups on the days when diet without steroids was given. Control and antiandrogen groups ate a consistent percentage of diet (approximately 7.2%) regardless of pill cycle date. Consequently, the actual dose provided for the three treated groups was lower than estimated by approximately 28% in the OC group, 26% in the OC+MT group, and 16% in the antiandrogen group.

Steroids

The OC and OC+MT groups had significantly lower serum levels of endogenous E2 ($p < .05$) than the control and antiandrogen groups. As expected, EE levels were significantly higher ($p < .001$) in both OC and OC+MT groups. Major treatment group differences could not be explained by day of sampling because we obtained similar measures for the OC and OC+MT groups when separated by day 2 or 3 of cycle. The serum levels of T were significantly lower ($p < .05$) in the OC compared with control and antiandrogen groups and were highest in the OC+MT group, presumably owing to cross-reactivity of MT in the testosterone assay (Table 3).

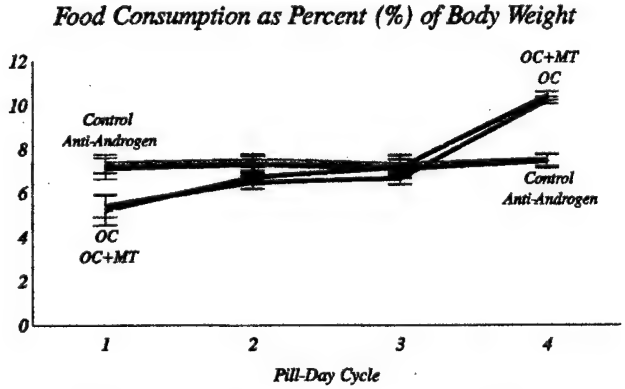

Figure 4. Average food intake for the duration of the experiment expressed as a percent of body weight. Mean ± SEM for each day of the pill treatment cycle for each treatment group is provided. OC- and OC+MT-treated animals were treated for 3 days on (days 1, 2, and 3) and 1 day off (day 4); antiandrogen treatment was daily.

Histopathology and Cell Counts

Blind subjective evaluation of H&E slides suggested that OC+MT animals had minimal mammary gland tubuloalveolar proliferation. Subjective histologic score (0 = minimal; 1 = mild; 2 = moderate; 3 = marked) was significantly different among groups ($p = .003$). Histologic scores were highest in the control group, followed by the antiandrogen group, OC group, and, finally, OC+MT group (1.64 ± 0.2, 1.17 ± 0.2; 1.07 ± 0.2, 0.50 ± 0.1, [mean ± SEM], respectively). Post hoc Tukey test indicated that OC+MT animals had significantly lower ($p < .05$) histologic scores compared with control animals. No tumors or inflammation were observed in any mammary glands.

Histomorphometric data are summarized in Table 4. Regardless of immunostaining, the area required to count up to 100 cells was higher in the OC+MT group than in the control and OC groups. The number of cells per unit area was lowest in the OC+MT and antiandrogen groups compared with the control and OC groups. These two measured values support the subjective cell density observed during histopathologic evaluation.

The number (with or without area adjustment) and percentage of PgR-positive cells were significantly highest ($p < .05$) in the OC+MT group compared with the other three groups. The number (with or without area adjustment) and percentage of PCNA-positive cells were significantly lowest ($p < .05$) in the OC+MT group compared with the other three groups.

DISCUSSION

In contrast to our previous study of OC effects in cynomolgus monkeys, the rats in this study did not eat the experimental diets as expected.[28] Manoharan et al also used diet as the method for OC delivery, which led to less food consumption and lower BW.[29] Interestingly, Liu and Lin found that subcutaneous delivery of OCs also led to lower BW.[30] Regardless of

Table 3. Serum Levels of Sex Steroids Measured at Necropsy for Treatment Groups

	Control	OC	OC+MT	Antiandrogen	*p*
E2 (pg/mL)					
Mean ± SEM	12.9 ± 1.9	6.9 ± 1.3	5.2 ± 1.0	9.6 ± 1.6	.01 Con > OC, $p < .05$ Con > OC+MT, $p < .05$
EE (pg/mL)					
Mean ± SEM	7.7 ± 3.1	67.8 ± 10.3	66.4 ± 8.8	5.7 ± 2.7	<.001 Con > OC, $p < .001$ Con > OC+MT, $p < .001$
T (ng/mL)					
Mean ± SEM	168 ± 22	121 ± 11	410 ± 21*	208 ± 30	<.001 Con < OC+MT, $p < .001$

Values are mean ± standard error of the mean. Data were analyzed by ANOVA, and post hoc comparisons between the control group and treatment groups were made using the Bonferroni method. Characteristics of individual radioimmunoassays are described in Materials and Methods.

* Cross-reactivity of MT in the T radioimmunoassays probably accounts for part of the high levels of T observed in the OC+MT group.

cause, ie, lack of appetite and/or food aversion, BW was significantly reduced in the OC and OC+MT groups. Unfortunately, it is not possible to determine the absolute role that alterations in BW have in the effect of the OC or OC+MT treatment on mammary tissue. However, because OC treatment caused greater decreases in food consumption and BW, and OC+MT treatment caused greater effects on mammary PgR and PCNA immunostaining, the effect of treatment cannot be explained by BW or food consumption alone.

Another relevant issue is how lack of food intake affected dosage. As demonstrated in Table 2, all three treated groups, OC, OC+MT, and antiandrogen, received a lower steroid dose than expected. However, by hormone analysis (Table 3), the OC and OC+MT groups received sufficient steroids in their diet to lower their circulating levels of endogenous E2 (central negative feedback), and the OC group received sufficient steroids in its diet to decrease its level of T (mild OC-induced hypoandrogenemia). We presume that the markedly elevated circulating levels of T in the OC+MT group were due to cross-reactivity of MT with the T assay.

The rationale for using MT in this experiment was based on logistical and scientific information. First, MT is commonly used as an oral androgen replacement drug.[31] Second, to our knowledge MT does not aromatize to estrogen.[14] Without aromatization, we prevent additional confounding factors such as artificial increases in estradiol. We could not measure SHBG in this experiment. A plasma T binding protein has been described in rats,[32] but SHBG is not present in rat tissues.[33]

The histologic changes and values measured in the OC animals were not significantly different from those of control animals. However, we were surprised by the histopathologic findings in the mammary glands of the OC+MT animals, which indicated decreased alveolar proliferation compared with control animals. This subjective histopathologic effect was supported by the low number of mammary epithelial nuclei per unit area and the immunologic findings in which the number of cells positively staining for PCNA was significantly decreased (Figure 5). Interestingly, the number of PgR-staining cells was highest in the OC+MT animals compared with the other three groups (Figure 6). Both an increase in

Table 4. Histomorphometric Evaluation of Mammary Gland Tissues Immunostained for Progesterone Receptor and Proliferating Nuclear Antigen

	Control	OC	OC+MT	Antiandrogen	ANOVA *p*	χ^2 *p*
PgR cell counts						
Area (mm^2)	0.96 ± 0.1	1.09 ± 0.1	1.42 ± 0.2	1.52 ± 0.2		.090
Total nuclei	129.5 ± 4.8	122.3 ± 3.3	122.1 ± 5.7	121.3 ± 3.6	NS	
%(−)	77.0 ± 1.5[a]	74.9 ± 2.3[a]	65.2 ± 2.5[b]	76.9 ± 2.3[a]	.001	
(−)/mm^2	115.2 ± 11.4[a]	95.5 ± 10.7[a,b]	74.3 ± 10.8[b]	73.0 ± 9.6[b]	.022	
%(+)	23.1 ± 1.5[a]	25.1 ± 2.3[a]	34.8 ± 2.5[b]	23.1 ± 2.3[a]	.001	
(+)/mm^2	33.3 ± 3.2[a,b]	30.6 ± 3.0[a,b]	40.4 ± 8.1[a]	20.8 ± 2.9[b]		.012
PCNA cell counts						
Area (mm^2)	0.81 ± 0.0	0.90 ± 0.1	1.02 ± 0.1	1.00 ± 0.0	.078	
Total nuclei	131.4 ± 6.8	127.7 ± 8.0	114.9 ± 2.2	125.5 ± 4.2	NS	
%(−)	95.4 ± 1.7	92.0 ± 2.9	98.8 ± 0.6	92.4 ± 2.6		.026
(−)/mm^2	156.8 ± 8.4	144.5 ± 15.9	118.2 ± 8.5	117.8 ± 7.1		.002
%(+)	4.62 ± 1.7	7.01 ± 2.9	1.20 ± 0.6	7.61 ± 2.6		.026
(+)/mm^2	9.28 ± 3.9	10.6 ± 4.2	1.26 ± 0.6	11.2 ± 4.0		.026

NS = not significant: $p > .10$.

Values include the total area measured to count 100 or more cells, total number of nuclei, and percentage and number per area of negative and positive cells. Analysis of variance *p* value is provided. Different letters indicate significant ($p < .05$) differences between groups, by post hoc Tukey test. Chi-square tests provide for nonlinear distributions.

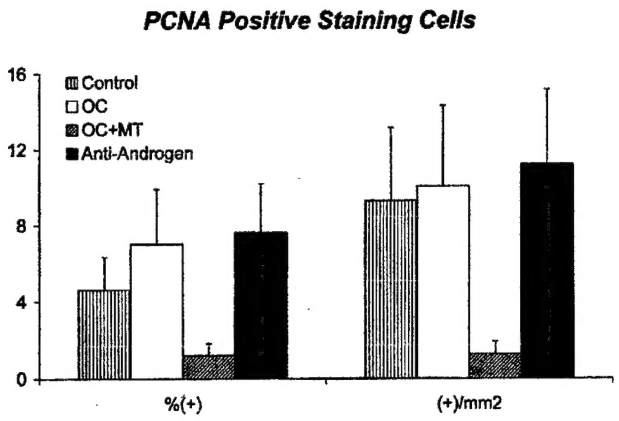

Figure 5. Bar graphs (mean ± SEM), by treatment group, showing data of PCNA-positive stained nuclei as a percentage of total nuclei, %(+), and as the number of positive nuclei per unit area, (+)/mm^2.

mammary epithelium PCNA positivity and PgR negativity have been associated with more aggressive breast cancer behavior in women.[34–36]

A possible explanation for the changes observed in tissue proliferation and PgR expression is that the addition of MT to OCs may have affected mammary epithelium biology through the AR. ARs are found in normal epithelium as well as in epithelia of mammary cancers.[4] However, the mechanisms by which androgens modulate normal or abnormal breast cell growth are not understood. To our knowledge, no publication has described the relative and/or combined contribution of estrogen plus progestin plus androgen on breast cancer cell or normal mammary gland epithelial cell proliferation in vitro. Some in vitro work suggests beneficial effects of androgens. It has been reported that androgen effects on breast cancer cell DNA synthesis may involve both genomic and nongenomic mechanisms.[37] When estrogen-responsive ZR-75-1 cells were grown in the presence of DHT alone or in combination with E2, DHT caused a marked down-regulation of Bcl-2 protein and messenger RNA levels.[38] Although proliferation of MFM-223 and ZR-75-1 cells is inhibited by androgens, addition of

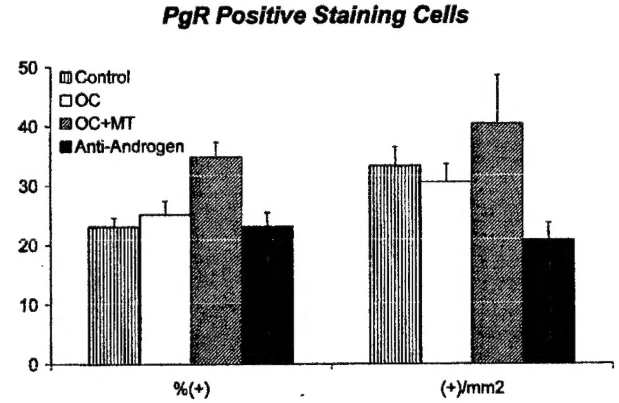

Figure 6. Bar graphs (mean ± SEM), by treatment group, showing data of PgR-positive stained nuclei as a percentage of total nuclei, %(+), and as the number of positive nuclei per unit area, (+)/mm^2.

10 nmol/L DHT stimulated the expression of gross cystic disease fluid protein-15 (GCDFP-15) mRNA in MFM-223 as well as the secretion of GCDFP-15 into the culture medium. GCDFP-15 is a major protein component of benign breast cysts and is observed in 50% of cancers.[39]

Progesterone inhibits the proliferation of normal breast epithelial cells in vivo as well as breast cancer cells in vitro, and the antiproliferative activity of progesterone in breast cancer cell lines may be due to its ability to induce apoptosis through Bcl-2 down-regulation and p53 up-regulation.[40] Studies of human breast carcinomas have shown that high PCNA immunoreactivity and absence of PgR are correlated with a shorter relapse-free period, and poorer prognosis and overall survival.[35,41,42] In fact, absence of PgR expression in primary breast cancer is associated with disease progression and may be a marker of an aggressive tumor phenotype.[43] Another potential explanation for the changes observed in the mammary gland of OC+MT animals is a potential synergistic effect between MT and the levonorgestrel contained in the OC preparation, especially because in the OC-treated group, which also received levonorgestrel, we found no differences in histopathology or immunohistochemistry compared with the control animals.

The balance of cell kinetic events (proliferation and apoptosis) in mammary tissue provided by all three hormones is undefined, but our results combined with other recent work suggest that some fundamental insights will be forthcoming. Recently, investigators have developed an animal model of mammary gland carcinogenesis using a combination of E2 and T in female Noble rats.[44] In their analysis, those investigators suggested that androgens might work as a promoter, shortening the latency time of mammary gland carcinogenesis. In addition, they found that animals implanted with estrogen or T alone also developed mammary cancers, although with a lower overall incidence than the two hormones combined. However, those investigators did not indicate how much or whether local conversion of T to E2 occurred in the experiment or whether endogenous progesterone levels were affected.

Because administration of antiandrogens such as bicalutamide improves the clinical symptoms of male patients with benign prostatic hypertrophy and cancer, we used bicalutamide in this experiment as a means to reduce the androgen activity in mammary tissues without having to administer OCs or surgically or chemically castrating the animals. The dose of bicalutamide (Casodex) provided by the diet in this experiment was probably lower than the recommended dose of 50 mg/day for a man with prostatic disease, and was well below the toxic levels described in rodents (approximately 2000 mg/kg of BW).[45,46] We did not measure the circulating levels of bicalutamide to confirm that, but the Casodex dose provided in the diet did not affect the circulating levels of endogenous steroids compared with control animals.

In our experiment, the antiandrogen-treated group had a suppression of PgR-positive staining cells per unit area and a

slight increase in PCNA-positive labeling. The significance of these changes is unknown.

Most of the literature available indicates little effect of OCs on the risk of breast cancer.[47,48] For example, OC use cannot explain the elevated risk of breast cancer observed in Asian women who have migrated to the United States.[49] Although women who reported using OCs for 10 or more years have no increase in incidence,[48] there is a suggestion that recent use is associated with an increased risk of breast cancer.[50]

Extrapolation of results from animal experimentation to human conditions requires careful consideration and evaluation. However, the present study suggests that the addition of an androgen to a low-dose OC may provide additional noncontraceptive benefits for women. Also, this benefit may potentially be extended to postmenopausal women who are interested in including androgen replacement therapy in the traditional hormone replacement therapy (HRT) (estrogen plus progestin). In a recent paper by Schairer et al,[51] a total of 46,355 postmenopausal women were evaluated to determine whether postmenopausal HRT using a combined estrogen–progestin regimen increased the risk of breast cancer beyond that associated with estrogen replacement therapy (ERT) alone. The data suggested that HRT increased breast cancer risk beyond that associated with ERT alone. However, in their publication there was no mention of users of androgen plus estrogen products (such as Estratest; Solvay Pharmaceuticals, Marietta, GA) or whether patients taking such products were included in either category. The dose, alkylation, and route of administration of an androgen such as MT in combination with HRT each may affect the occurrence of potential side effects in women; however, the lower doses of androgens already used in women appear to have minimal side effects.[31]

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